Comparison of serum and synovial fluid markers of Herpes simplex virus and *Helicobacter pylori* infection between rheumatoid arthritis and osteoarthritis patients: A Retrospective Case-Control Study

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Recently, several infectious agents including Epstein-Barr virus and Escherichia coli have been suggested as possible contributing factors to the pathogenesis of rheumatoid arthritis (RA). This study was designed to compare serum and synovial fluid markers of herpes simplex virus (HSV) and *Helicobacter pylori* of RA and osteoarthritis (OA) patients.

This comparative study was conducted on two hundred OA and RA patients who referred to the Rheumatic Diseases Research Center (RDRC) affiliated with Mashhad University of Medical Sciences, Mashhad, Iran, from March 2015 to 2016. Synovial fluid was obtained from all individuals. Two years later, participants attended a follow-up session to collect blood samples for serum markers of these two infectious agents.

Twenty-five patients (96.15%) in the RA group and 23 individuals (92%) in the OA group had positive serum IgG antibodies for HSV. As for *Helicobacter pylori*, 13 individuals (50%) in RA and 12 individuals (48%) had positive serum IgG antibodies (*p* value = 0.66). In addition, 9 (34.6%) and 8 (30.8%) in the RA group and 10 (40%) and 3 (12%) in the OA group had positive serum IgA and IgM antibodies for *Helicobacter pylori*, respectively (*p* value = 0.89 and *p* value = 0.13, respectively). Collected fluid samples were negative for both *Helicobacter pylori* and HSV1 and 2 DNA particles in all individuals.

Based on the results of the current study, there is no difference between RA and OA patients in terms of Herpes simplex virus and *Helicobacter pylori* infection.

**Keywords:** Helicobacter pylori, Herpes simplex virus, Osteoarthritis, Rheumatoid arthritis, Synovial fluid

**Introduction**

Rheumatoid arthritis (RA) is one of the most common inflammatory conditions of unknown etiology, characterized by symmetrical peripheral polyarthritis [1, 2].

Several infectious agents have been suggested as potential contributing factors in the development of RA, including both bacterial (i.e. *Helicobacter pylori* and Mycoplasma pneumonia) and viral (i.e. Cytomegalovirus, Epstein-Barr virus, Parvovirus B19, and retroviruses) agents. Immune complexes formed as a result of immune reactions to infectious agents lead to an induction of RA-related factors. One of these is the rheumatoid factor (RF), which has a high affinity to the Fc region of immunoglobulins [3].

Additionally, infection results in an increased permeability of the synovial membrane to leukocytes, which leads to their accumulation and an increased expression of adhesion molecules (i.e. integrins, selectins, laminins, and fibronectins) and immunoglobulins. Gene expression of inflammatory cytokines is also increased, leading to neoangiogenesis in tissue [4, 5]. These changes, together with activation of regional fibroblasts, cause the formation of inflammatory structural changes similar to those seen in RA [6].

Several epidemiologic studies have shown an association between periodontal diseases and RA [7-9]. In addition, Porphyromonas gingivalis and Escherichia coli, both of which are involved in infectious periodontal diseases, also play roles in the production of autoantibodies, citrullination of peptides, and synovial inflammation [10].

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Viral agents, alone or together with bacterial pathogens, can either directly or indirectly through induction of autoimmune reactions damage joints. Several previous studies have evaluated the association between H. pylori and the development of RA, and some of them established an association between H. pylori infection and elevated disease markers of RA [11]. Nonetheless, a cohort study with a relatively large sample size found no difference in the risk of acquiring RA between H. pylori positive and negative patients in a follow-up period of 8 years [12].

Due to the controversial findings in the literature on the relationship between H. pylori infection and autoimmune disease, this study aimed to compare the serum markers of infection with Herpes simplex virus 1 (HSV-1) and Helicobacter pylori between RA patients and OA patients.

Materials and Methods

This comparative study was conducted on OA and RA patients who referred to the Rheumatic Diseases Research Center (RDRC) affiliated with Mashhad University of Medical Sciences, Mashhad, Iran, from March 2015 to 2016.

Inclusion and Exclusion Criteria

The inclusion criteria were age over 18 years and a diagnosis of grade 3 or 4 OA based on the Kellgren and Lawrence criteria or RA established by a rheumatology subspecialist based on the American College of Rheumatology criteria, 2010. Following inclusion, patients suspected of having septic arthritis, had crystals on microscopic evaluation of their synovial fluid, or had consumed any form of antibiotic during the two weeks prior to sampling as well as patients with no available medical records or a history of herpes labialis during the 3 months prior to sampling were excluded.

Study Design

This study was performed on all OA and RA patients who referred to RDRC from March 2015 to March 2016. In general, 200 patients (100 established RA cases and 100 OA patients) were randomly selected to participate in this study. The data was gathered by a researcher-made questionnaire covering demographic information which was completed for each case separately. Synovial fluid aspiration (2-5 milliliters) was performed by a rheumatology subspecialist using sterile aspiration needles and sterile tubes with no additives, and the samples were stored at -20 °C under appropriate laboratory conditions to be analyzed later.

According to the results of the evaluation of medication consumption, the RA patients consumed prednisolone (83%), methotrexate (73%), hydroxychloroquine (33%), sulphasalazine (26%), and biologic medicines (9%) in descending order. Considering the disease duration, the results revealed no significant difference between the two groups in this regard (p value = 0.07).

Arthrosynthesis had been performed in RA and OA patients due to knee effusion in 2015 to 2016. These patients required synovial fluid drainage as part of their treatment process. Drainage of joint fluid was performed by a rheumatologist considering the principles of aseptic technique.

All synovial fluid samples were evaluated for crystalopathies and infections, and suspicious cases were excluded from the study. The remaining samples were centrifuged at 12000 g for 30 minutes and stored at -20 °C. All laboratory operators were blinded to clinical diagnoses. In 2018, 26 individuals from the RA group and 25 individuals from the OA group attended a follow-up session during which venous blood samples (50-10 milliliters) were taken by a laboratory operator using sterile needles. The obtained samples were centrifuged at 1200 g for 10-15 minutes and stored at -20 °C to be later analyzed for serum markers of infection.

Laboratory analyses

Real-time PCR (RT-PCR) for HSV1 and 2 and H. pylori's DNA was applied to remove the viscosity of synovial fluid. The samples were washed by phosphate buffered saline (PBS), and then the DNA was extracted using a Genetbio® commercial kit (South Korea) according to the manufacturer's instructions. After DNA extraction, RT-PCR was performed using a Fanavari Novin kit (Tehran, Iran) according to the manufacturer protocol. The master mix was inserted in designated microtubes. For PCR cycling, 30 μl of Master Mix was added to 10 μl of the isolated DNA. A thermocycler was used to amplify the DNA content of the samples in PCR cycling conditions: denaturation phase (at 98 °C for 15 seconds), annealing phase (at 63 °C for 60 seconds), and extension phase (at 72 °C for 120 seconds). This cycle was repeated 60 times. Following that, Agarose gel electrophoresis was used to detect and quantify the DNA contents. Finally, the data was analyzed using Rotor-Gene 6000 software [13].

Enzyme-linked immunosorbent assay (ELISA) for serum markers of HSV1 and 2 and H. pylori infection was performed using a Pishat Zah Zaman Diagnostics (Tehran, Iran) ELISA kit. 10 μl of serum sample was mixed with 1 milliliter of diluting solution, and 100 μl of the mixture was added to each well on the ELISA plate. Standard, control, and sample dilutions were added to their designated wells. Following that, the plate was incubated at room temperature (22-28 °C) for 30 minutes. After the incubation period, each well was emptied and then washed using detergent solution five times. Immediately following that, 100 μl of antibody-enzyme conjugate. The ELISA plate was then incubated at room temperature (22-28 °C) for an additional 30 minutes. Then the washing process was performed five more times. 100 μl of Colorimetric substrate was added to each well and the plate underwent 15 minutes of incubation at room temperature (22-28 °C) in a dark environment. Immediately after that, 100 μl of stop solution was added to each well, and the resulted mixture was analyzed using the ELISA reader machine at the wavelength of 450 nanometers and using a reference wavelength of 630 nanometers. Finally, the standard curve was used for determining the concentration of the provided samples.
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**Statistical Analysis**

Statistical analyses were carried out using IBM-SPSS software version 21. The normality of the data was assessed using the Kolmogorov-Smirnov test. After the normality of data was calculated, the t-test or its nonparametric equivalent test (i.e. Mann-Whitney U test) was employed to analyze the variables. Moreover, the chi-square and Fisher exact test were used to analyze the data. A *p* value less than 0.05 was considered statistically significant.

### Results

This study enrolled 200 patients, and the majority of them were female (*n* = 161, 80.5%). RA and OA groups included 77 (77%) and 23 (23%) and 84 (84%) and 16 (16%) females and males, respectively, which showed no significant difference between the two groups in terms of gender (*p* value = 0.21). **Table 1** summarizes the mean ages and durations of illness of the two groups. Regarding age, 91% of the OA patients were more than 50 years old, and the patients in the OA group were significantly older than those in the RA group (*p* value < 0.05).

**Table 1.** The mean of age and duration of illness between two groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>RA group</th>
<th>OA group</th>
<th><em>p</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (years)</td>
<td>51.91</td>
<td>61.37</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>SD</td>
<td>14.92</td>
<td>9.85</td>
<td></td>
</tr>
<tr>
<td>Duration of illness (years)</td>
<td>8.41</td>
<td>7.07</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>6.28</td>
<td>3.73</td>
<td></td>
</tr>
</tbody>
</table>

Serological data of RA patients revealed that the mean erythrocyte sedimentation rate (ESR) and anti-CCP were 34.18 ± 2.57 mm/h and 122.10 ± 1.59 IU/ml, respectively. Furthermore, the anti-CCP parameter (82%) and ESR (58%) rates were higher than normal, and RF was positive in 77% of patients in the RA group.

The PCR analysis of joint fluid in both groups showed that all samples were negative for herpes virus DNA; however, two of the synovial fluid samples were found to be positive for *Helicobacter pylori*.

**Table 2** tabulates the mean concentration values of HSV IgG in the patients. According to the results, there is no statistically significant difference between the RA and OA groups (*p* value = 0.66), RF-negative and -positive groups (*p* value = 0.21), positive and negative anti-CCP groups (*p* value = 0.2), or positive and negative ESR groups (*p* value = 0.13) in terms of HSV IgG level. Furthermore, female RA patients and their OA counterparts (*p* value = 0.67) showed no statistically significant difference with male RA patients and their OA counterparts (*p* value = 0.82).

**Table 2.** The rate of IgG Herpes virus in patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean±SD</th>
<th>t-test</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups</td>
<td>RA</td>
<td>OA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>176.83±86.88</td>
<td>187.83±86.27</td>
<td>-0.443</td>
</tr>
<tr>
<td>Gender</td>
<td>Men</td>
<td>RA</td>
<td>OA</td>
</tr>
<tr>
<td></td>
<td>187.44±70.95</td>
<td>197.73±32.85</td>
<td>174.30±91.62</td>
</tr>
<tr>
<td>RF</td>
<td>Positive</td>
<td>166.36±83.22</td>
<td>202.69±67.45</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>220.80±97.75</td>
<td>202.69±67.45</td>
</tr>
<tr>
<td>ESR</td>
<td>Positive</td>
<td>166.36±83.22</td>
<td>202.69±67.45</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>220.80±97.75</td>
<td>202.69±67.45</td>
</tr>
</tbody>
</table>

**Table 2** tabulates the mean concentration values of HSV IgG in the two groups. According to the results, there is no statistically significant difference between the RA and OA groups (*p* value = 0.66), RF-negative and -positive groups (*p* value = 0.21), positive and negative anti-CCP groups (*p* value = 0.2), or positive and negative ESR groups (*p* value = 0.13) in terms of HSV IgG level. Furthermore, female RA patients and their OA counterparts (*p* value = 0.67) showed no statistically significant difference with male RA patients and their OA counterparts (*p* value = 0.82).
Other results showed that the positive H. pylori’s serum IgG condition had no statistically significant difference between the two groups (p value = 0.82). No statistically significant difference was observed between the two groups in terms of H. pylori’s serum IgA (p value = 0.89) or serum IgM (p value = 0.13). Table 3 presents the two groups in terms of positive H. pylori’s serum IgG, IgA, and IgM.

Discussion

Based on the PCR analysis of joint fluid in the RA and OA groups, all samples were negative for herpes virus DNA; however, two of the synovial fluid samples were found to be positive for Helicobacter pylori. The current study shows that there is no significant difference between RA and OA patients for infection with HSV1, 2 or H. pylori.

Due to the lack of basal membrane in the synovium or its damage in patients with rheumatoid arthritis and osteoarthritis, these people are prone to infection with HSV [14]. On the other hand, the proliferation of microbes affects the immune system response and the release of inflammatory mediators, including interleukin-3 and TNF-alpha, leading to cartilage, synovitis, and joint destruction [15]. In a large cohort study by Bartels et al., the researchers evaluated a total of 56,000 patients for H. pylori using the urea breath test (UBT); these patients also underwent a follow-up period of 8 years. Their results indicated that there was no significant difference in terms of incidence of new RA cases between H. pylori positive and negative patients during the course of the follow-up period [12]. Saad et al. demonstrated no significant clinical or laboratory differences between Helicobacter pylori positive and negative patients in terms of rheumatoid [16]. Similarly, no difference was reported between RA and OA patients with Helicobacter pylori positive and negative findings regarding the clinical and laboratory results, which is consistent with the results of the present study.

The prevalence rate of Helicobacter pylori in autoimmune diseases was not remarkable, as it was found that Helicobacter pylori infection increased with age [17]. In this study, the majority of OA patients were more than 50 years old, and data analysis showed that the patients in the OA group were older than those in the RA group.

The current study also showed that the presence of HSV DNA in the synovial space in one-third of the patients with RA could be due to the patient’s temporary exposure to HSV. Additionally, the presence of HSV DNA in joint fluid indicates that the DNA of this virus has entered the space and joint fluid through white blood cells from the systemic bloodstream. HSV DNA was negative in the joint despite the positive blood level, which may be due to the distance between sampling, the consumption of antibiotics, or other detectable variables.

The probable effect of HSV infection as an independent factor on the development of immune system disorders, inflammation, and autoimmunity was not remarkable in the current study. Generally, the PCR of HSV types 1 and 2 was not positive in any of the joint fluid samples of the patients with RA and OA in the current study.

Similarly, no trace of the herpes virus was reported in joint fluid or serum samples of the OA patients in the above-mentioned study; therefore, it can be surmised that both studies were conducted in the same method.

The present study showed no difference between the RA and OA groups. Burgos et al. showed that IgG and IgM levels were above normal in 50% of RA patients. Furthermore, no significant difference between the level of antibody and PCR was reported in the analysis of IgG and IgM antibodies against HSV in serum and joint fluid [18]. Larionova et al. utilized serological methods to study IgG and IgM, and their results indicated that increased IgM anti-human herpes virus response in RA patients is related to HSV1/2 reactivation [19].

H. pylori infection as a stimulating and intensifying factor in autoimmune diseases was confirmed by Smyk et al. [20]. There is evidence on the cross reactivity between T-cells and the production of autoantibodies in autoimmune disease patients with H. pylori infection [21]. Furthermore, Kandil et al. studied idiopathic juvenile arthritis patients and found that compared with healthy children, patients with juvenile idiopathic arthritis had a 34.1% greater chance of being seropositive for H. pylori [22].

It is of paramount importance to mention that H. pylori can be an exacerbating factor for RA or related conditions. It is hypothesized that H. pylori infection leads to aggravating symptoms and inflammation in autoimmune diseases. For example, in a recent study performed on 100 RA patients by Ebrahimi et al., researchers found that H.
H. pylori seropositive RA patients had significantly higher serum levels of rheumatoid factor (RF), ESR, CRP, anticyclic citrullinated peptide (Anti-CCP), and anti-mutated citrullinated vimentin (Anti-MCV) than their seronegative counterparts. Moreover, they found that H. pylori seropositive RA patients who also had a positive cytotoxin-associated gene A protein (CagA) also scored significantly higher on disease activity score 28 (DAS28) and visual analogue scale (VAS) than their CagA negative counterparts [23]. Jones et al. estimated the presence of H. pylori infection to be 43–68% among RA patients, [24] which is consistent with the findings of the study performed by Ebrahimi et al. [23]. Zentilin et al. confirmed the results of the aforementioned study on the higher rate of follow-up after the commencement of therapy for patients with H. pylori infection in comparison to those who are not infected by H. pylori [25].

It should be noted that development of systemic rheumatic disease is not dependent exclusively on an infectious agent. Therefore, H. pylori infection may be only an underlying factor for the expression of particular genes leading to autoimmune diseases. This might be due to an interaction between the infectious agent and a cascade of host-specific factors [11].

According to the results of the current study, however, it is more probable that Helicobacter pylori has no obvious effect on RA and OA prevalence or incidence. Although the sufficiency of the follow-up length is debatable, the results show that infection with Helicobacter pylori is unlikely to be a risk factor for the development of RA, which is consistent with the results of the current study.

The key limitation of the current study was the lack of follow-up after the commencement of H. pylori eradication therapy to assess the clinical outcomes of RA patients who were seropositive for this agent. The other limitation of this study was the lack of adjusting the effects of age on the comparisons of the serum markers of infection with Herpes simplex virus 1 (HSV-1) and Helicobacter pylori between RA and OA patients. The authors suggest that future studies include a follow-up in long enough intervals after eradication therapy to evaluate how RA patients have responded when compared to their baseline.

**Conclusion**

Based on the results of the current study, there is no difference between RA and OA patients in terms of Herpes simplex virus and Helicobacter pylori infection. The results further suggest that because the difference in serum marker levels and OA patients is not significant and that the DNA particles of both agents are absent in the synovial fluid of RA patients, it is unlikely that either HSV1, 2, or H. pylori is a risk factor for the development of RA.

**Declarations**

**Ethical Considerations**

This study was approved by the Ethics Committee of Mashhad University of Medical Sciences, faculty of medicine which can be accessed using the code “IR.MUMS.sm.REC.1395.614”. All patients filled out an informed consent form and were free to leave at any point during the study. All of the collected data were kept confidential and the patients’ names were excluded from the data. None of the authors of this study had conflict of interest or financial ties to disclose.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Funding**

None.

**Author Contributions**

This work was carried out in collaboration among all authors. MS designed the study, YS performed the statistical analysis, HR performed Laboratory analyses, MKH wrote the protocol, and wrote the first draft of the manuscript. SS and MG managed the analyses of the study. HH and KH managed the literature searches. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

**Acknowledgments**

Declared none.

**Conflict of interest statement**

The authors declare that there is no conflict of interest.

**References**


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